CHAPTER 4
endocytosis of gold nanoparticles
4.1 Introduction

Cellular uptake of materials and macromolecules through plasma membrane of the cells is known as endocytosis. Endocytosis is classically divided into phagocytosis i.e. ingestion of particles with a diameter more than 0.1 µm, and pinocytosis i.e. ingestion of particles or molecules less than 0.1 µm [1]. Phagocytosis is the process by which cells engulf foreign particles occurs in all eukaryotes. The term phagocytosis was coined by Metchnikoff and is derived from the Greek “phagein” (eating) and “cytos” (the cell) i.e. cell eating. The unicellular heterotrophic organisms, such as amoeba and ciliates use it for nutrition by trapping food particles and smaller organisms and enclosing them within folds of plasma membrane. The folds form vacuole (or phagosome) that pinches off from the plasma membrane and later on fuses to lysosome, and the material digested intracellularly.

In mammals, phagocytosis plays a protective role [2], rather than a mode of feeding. Mammals possess a variety of phagocytic cells, including macrophages and neutrophils, whose function is to patrol through the blood and tissue, phagocytizing invading organisms, damaged cells, aging red blood cells and debris. The phagocytosis is initiated by the binding of particle ligand to a variety of receptors at the cell surface. This triggers an array of signals that lead to rearrangements of the actin cytoskeleton.

In a series of biochemical events synthesis of phagosome takes place. In order to kill the microbe and degrade the engulfed material, fusion of phagosome with lysosomes takes place and results into the formation of phagolysosome.

Pinocytosis (cell drinking) is further divided according to whether the cell ingests substances that are in the fluid phase or bound to the pinosome membrane. The former type of endocytosis is called fluid phase endocytosis and may occur by two pathways, uptake of fluid within either macropinocytic vesicles (macropinocytosis), which is associated with the formation of relatively large vacuoles that are heterogeneous in size and shape (0.5-200 nm), and micropinocytosis which occur through the generation of small vesicles (50-150 nm) [3]. In this case the ingested substance nonspecifically binds to the membrane. With both micropinocytosis and macropinocytosis, solute uptake is directly proportional to the volume of fluid internalized and solute concentration. There are evidences that
micropinocytosis also occurs via more than one pathway. In addition to clathrin-dependent endocytosis, which is responsible for the internalization of most, but not all, known receptors and their ligands (receptor-mediated endocytosis), other less-well characterized pathways also exist, at least in some of the cell-types, and are collectively referred to as clathrin-independent endocytosis. Clathrin-dependent endocytosis is responsible for the internalization of most, but not all, cell surface receptors that have been characterized, including receptors with housekeeping functions that cycle constitutively between endosomes and the plasma membrane, and receptors for growth factors and hormones whose entry process is regulated by ligand-binding. Once formed, clathrin-coated vesicles specifically dock onto and fuse with early endosomes, thereby delivering their membrane and content. Many components regulating protein traffic and sorting in this pathway have been identified and characterized, in some cases at the atomic level, including the adaptor complex 2 (AP2) [4]. Much less is known about clathrin independent endocytosis, which itself may comprise of different mechanisms. This route is involved in retrieval of membrane after induced exocytosis in adrenal chromaffin cells [5] and is responsible for the uptake of desmosome [6]. Interestingly, recent studies revealed that the interleukin 2 receptor (IL2-R) is internalized via a pathway which does not depend on Eps15, a key-component of the clathrin pathway but might involve cell surface micro domains rich in cholesterol and glycosphingolipids (“rafts”) [7]. Several lines of evidence, indeed, suggest that such micro domains can provide an additional entry route, in particular caveolae, which contain the protein caveolin, at least in some cell-types [8]. In addition, internalization might also occur via separate pathway(s) that does not depend on clathrin/AP2 or rafts/caveloae [9].

It is generally believed that that clathrin-dependent and -independent routes then meet in early endosome, the first station of the endocytic pathway, consistent with the fact that IL2-R follows the classical degradation pathway to lysosomes. However, it is also possible that other entry routes by-pass early endosomes. SV40 was recently shown to enter cells via caveolae and then to reside within caveosomes that do not contain endocytic tracers [10]. Whether this pathway is a specialized, virus-induced route of entry remains to be investigated.

Both phagocytosis and macropinocytosis are triggered in specialized cells in response to specific stimuli. Macropinocytosis is a non-clathrin route of entry related
to phagocytosis and associated with areas where membrane spreading and ruffling takes place. This route is modulated and used by some pathogenic bacteria [11]. It is stimulated by the epidermal growth factor EGF [12], and by Ras [13]. Macropinocytosis involves remodeling of the cortical actin cytoskeleton, involving Ras, Rac, and cdc42 [14] and Rho proteins, implicated in macropinocytosis down regulation in dendritic cells during maturation [15]. After internalization into animal cells, cell surface proteins and lipids, as well as solutes, first appear in peripheral early endosomes, sometimes referred to as sorting endosome [16]. Depending on their fate, these proteins can either be recycled back to the cell surface for reutilization, at least in part via recycling endosomes, or transported to late endosomes and then lysosomes to be degraded. The recycling and lysosomal pathways exhibit major differences with respect to membrane organization and dynamics. Lysosomes are intracellular organelles surrounded by a membrane containing various hydrolytic enzymes usually requiring acidic pH for optimal function. Except mature erythrocytes, lysosomes are present in all mammalian cells. Normally in cells, at any point of time there are two types of lysosomes present in the cells viz. primary lysosomes and secondary lysosomes. Primary lysosomes are the lysosomes which have not yet come into the contact with endocytosed material. Their pH is neutral. Secondary lysosomes are actually lysosomes fused with phagosome or endosome. Their pH is acidic and they contain various amount of ingested material at various stages of degradation. One more term tertiary lysosomes has also been introduced which refer to residual bodies that contain undegradable components of engulfed material.

Substances entering the lysosomes are broken down to small molecules which then are transported across the lysosomal membrane into the cytoplasm. There they are metabolized for biosynthesis processes. In some cases discrepancy in the digestion process leads to lysosomal overloading. This may happen, e.g. after ingestion of synthetic polymers or metal nanoparticles against which lysosomal enzymes are lacking, or after engulfment of microorganisms that block fusion of phagosome with lysosomes either because of their structure or secretory products.

Hence, from the above description it is clear that the routes of entry to the cells are quite complex and may be, one or more type of endocytosis may work simultaneously in case of nanoparticles. Although, there are many reports on the
uptake of nanoparticles by the cells [17-28], our knowledge of their mode of cellular internalization is limited due to their shape, size, composition and surface functionalization related complexities and varieties.

Macrophage endocytosis of superparamagnetic iron oxide nanoparticles (which are already used in cellular biochemical pathways) has recently been shown [29]. In order to ascertain the endocytotic pathway of gold nanoparticles, in the present work various microscopy techniques such as atomic force microscopy (AFM), confocal laser scanning microscopy (CFLSM) and transmission electron microscopy (TEM) were used to trace the time-dependent mechanism of gold nanoparticle uptake inside the macrophage cells.

4.2 Scheme of work

The first part of the chapter emphasizes on the functionalization of preformed gold nanoparticles using lysine and poly-l-lysine amino acids. Since lysine doesn’t have a thiol group, it can stabilize the nanoparticles only through its amine group. Lysine capped gold nanoparticles were highly stable in solution as well as solid form, indicating that the stability is derived from the functionalization of nanoparticle surface with lysine. These capped nanoparticles were further functionalized with fluoresceine-iso-thio-cyanate (FITC). Later the interaction of gold with lysine and FITC has been discussed. The synthesized fluorescent gold nanoparticles were further characterized for cytotoxicity.

The second part of chapter deals with the study of time dependent kinetics of gold nanoparticle internalization inside the macrophage cells using different sophisticated microscopic tools viz AFM, CFLSM and TEM, and FACS. The early events of particle internalization tracked by AFM suggests pinocytotic mode of internalization. The mechanism of internalization of gold nanoparticles has been discussed using phagocytosis specific inhibitor cytochalasin B. The flow chart below describes the steps performed and materials and methods employed in this work.
4.3. Results and discussion

4.3.1 Physicochemical characterization of synthesized gold nanoparticles.

**UV-Vis absorption and Fluorescence emission spectroscopy studies**

UV-Vis spectra of the dialyzed borohydride reduced gold hydrosol, lysine (lys) and poly-L-lysine (PLL)-capped gold hydrosols and FITC (curves 1-4 respectively) are shown in Fig.4.1A. A strong absorption in curve 1 at ca. 527 nm is observed that corresponds to excitation of surface plasmon vibrations in the gold nanoparticles. When the gold nanoparticles are capped with lys or PLL, a broadening and red shift of the surface plasmon band is observed (curves 2 and 3 respectively) which indicates surface-complexation by the amino acid (lys) and the peptide (PLL) and hence, possibly some aggregation of the gold nanoparticles consequent to surface modification.
Figure 4.1 Physico-chemical characterization of gold nanoparticles by (A) UV-Vis absorption spectroscopy, (B) fluorescence emission spectroscopy. Curves 1-4 in A and B correspond to borohydride-reduced gold nanoparticles (curve 1), Au-lys (curve 2), Au-PLL (curve 3), and FITC (curve 4). Curves in B correspond to fluorescence emission spectra derived from the supernatants obtained after centrifugation of respective colloidal gold solutions using 10 μM FITC.

Interestingly, the spectrum recorded from Au-lys nanoparticles (curve 2) shows greater broadening and red shift in comparison with that of Au-PLL nanoparticles (curve 3). The interaction between aqueous gold nanoparticles with lys- and PLL thus appears to be quite complex. Solution containing FITC molecules shows an absorption maximum at ca. 456 nm. The solutions containing gold nanoparticles and lys- and PLL-capped gold nanoparticles were functionalized with fluorescein isothiocyanate (FITC). FITC (Fig.4.2) is a yellow-green coloured low molecular weight dye, can be conjugated to proteins via reaction with primary amine groups at high pH. The isothiocyanate group (\(-\text{N} = \text{C} = \text{S}\)) is reactive with amine groups on proteins inside cells. FITC is excitable at 488 nm and produces maximum fluorescence emission around 520 nm. The relative binding of FITC molecules to colloidal gold, Au-lys and Au-PLL were studied. Fig.4.1B shows the fluorescence emission spectra recorded from the supernatants obtained from 10 μM FITC-functionalized gold nanoparticles (curve 1), Au-lys (curve 2) and Au-PLL (curve 3) after centrifugation and from free FITC at the same concentration (curve 4). The decrease in the emission intensity of the above-mentioned solutions in comparison
with the FITC solution is a measure of the extent of binding of FITC molecules to the different gold nanoparticle solutions.

It is evident from the curves that the uncapped gold nanoparticles (curve 1) show maximum binding of FITC followed by Au·lys (curve 2) and Au·PLL (curve 3) respectively. In fact, the uncapped gold nanoparticles are observed to bind almost all the FITC molecules in the solution (curve 1) indicating the possibility that there might still some additional sites available for FITC binding on the gold nanoparticle surface. To completely functionalize the surface of gold nanoparticles with FITC molecules, a higher concentration of FITC (final concentration 50 μM) was used. Fig.4.3A in the shows the fluorescence emission spectra recorded from supernatants obtained from 50 μM FITC-functionalized gold nanoparticles (curve 1), Au·lys (curve 2) and Au·PLL (curve 3) after centrifugation and from free FITC in solution at the same concentration (curve 4). At higher concentration of FITC, trends similar to those observed at lower concentration of FITC (Fig.4.1B) are seen, i.e. uncapped gold nanoparticles (Fig.4.3A; curve 1) showed more binding of FITC that is followed by Au·lys (Fig.4.4A; curve 2) and Au·PLL (Fig.4.3A; curve 3) in that order. However, at the higher FITC concentration of 50 μM, the gold nanoparticle surface is saturated with FITC molecules, as is evident by the increase in emission intensity due to fluorescence from free FITC molecules in the solution (compare curves 1 in Fig.4.1B and Fig.4.3A). The quantitative estimation of the amount of FITC bound to various gold nanoparticles was done by plotting a standard calibration curve (Fig.4.3B) using 2.5
μM (point 1), 5 μM (point 2), 12.5 μM (point 3), 25 μM (point 4) and 50 μM (point 5) FITC concentrations, followed by linear curve fitting. The concentration of free FITC in nanogold solutions shown in Fig.4.3A was calculated using this standard calibration curve, which in turn gives the concentration of FITC bound to various gold nanoparticles. The amount of FITC bound to 100 μM Au, Au-lys and Au-PLL is found to be c.a. 1.33, 1.06 and 0.16 μM respectively. Since, the isothiocyanate group of FITC is a strong nucleophile, which is known to form stable complexes with gold [30], it is reasonable to expect that FITC binds to gold via the sulfur atom. Whereas in case of Au-lys and Au-PLL, due to non-availability of free gold surface for thiol linkage, electrostatic interaction between the amine and carboxylic groups of FITC and lys or PLL molecules on the surface of gold nanoparticles appears to be the major mode of linkage. The relatively higher concentration of FITC molecules bound to uncapped gold nanoparticles in comparison with Au-lys and Au-PLL nanoparticles can be explained based on the fact that the FITC thiol-gold interactions are relatively stronger than amine-gold interactions [31]. The higher binding of FITC molecules to Au-lys than Au-PLL can also be explained: the isothiocyanate moiety of FITC is well known to bind to the ε-amine group and N-terminal α-amine group of lysine residues [32]. While lys contains two free amine groups (α and ε), in the PLL chain, the α-amine group of each lys moiety is occupied in amide bond formation, except the N-terminal free α-amine group and hence only the ε-amine groups are free in PLL for binding. The presence of a larger number of free amine groups on the gold nanoparticle surface in case of lys rather than PLL could explain the higher binding of FITC molecules to lys rather than PLL.

**TEM and CFLSM studies**

The above-mentioned functionalized gold nanoparticles were observed under TEM and CFLSM. The TEM images recorded from as-prepared borohydride reduced gold nanoparticles, Au-lys and Au-PLL nanoparticles are shown in Figs.4.4A-C respectively. A comparison of the micrographs shows that while the average morphology of the particles is spherical, gold nanoparticles of various functionalities show varying degrees of particle aggregation. The as-prepared borohydride reduced gold particles are in close contact after water evaporation (Fig.4.4A) as is to be expected from the fact that they are not stabilized with an amino acid or a peptide.
The Au-lys (Fig.4.4B) and Au-PLL (Fig.4.4C) nanoparticles appear to be assembled into open, string-like structures and would explain the broadening and shift in the surface plasmon band observed in the UV-Vis measurements from these samples (Fig.4.1A). The extent of aggregation is maximum for Au-PLL (Fig.4.4C) suggesting a higher degree of inter-particle hydrogen bonding in this case. Fig.4.4D-F show TEM images recorded from the as-prepared borohydride reduced gold nanoparticles, Au-lys and Au-PLL nanoparticles respectively after further functionalization with FITC molecules. All these gold nanoparticle solutions functionalized with FITC show aggregation of gold nanoparticles as evident from the TEM images (Fig.4.4D-F).

Figure 4.4 Physicochemical characterization of gold nanoparticles (A-F) by transmission electron microscopy, and (I-K) confocal laser scanning microscopy. images obtained from borohydride-reduced gold (A), Au-lys (B), Au-PLL (C), Au-FITC (D), Au-lys-FITC (E), and Au-PLL-FITC (F). The inset in C shows the selected area electron diffraction pattern obtained from the gold nanoparticles G-I show CFLSM images obtained from solutions corresponding to those in D-F, respectively.
A comparison of these images indicates a relatively higher degree of aggregation in Au-PLL-FITC than in Au-lys-FITC followed by the Au-FITC nanoconjugates. The FITC conjugated Au-lys (Fig.4.4E) and Au-PLL (Fig.4.4F) nanoparticles also appear to be assembled into open, string-like structures of increasing lengths. These FITC conjugated borohydride reduced gold nanoparticles, Au-lys and Au-PLL nanoparticles were also observed under CFLSM, shown in Figs.4.4G-I respectively. As a control, FITC only, when seen under CFLSM, following the same washing steps as during sample preparation, did not result in any fluorescence under CFLSM. In the case of Au-FITC nanobioconjugates (Fig.4.4G), excited at 488 nm using an argon laser, even though we managed to observe fluorescence directly through the objective lens of the microscope, we could not adequately image the Au-FITC nanobioconjugates. This observation appears to be contrary to the fluorescence emission spectroscopy data (Fig.4.1B) in which the FITC-gold nanoparticles show good fluorescence emission. This inability to image the FITC-capped gold nanoparticles by CFLSM indicates rapid quenching in fluorescence intensity from Au-FITC nanoparticles (Fig.4.4G). Quenching of the excited state by the conductive metal surface that result in energy transfer to the metal surface has been reported earlier [33-34]. The probability of this Forster energy transfer depends on the overlap of the fluorescence band of dye molecule with the absorption band of the acceptor [34]. In the present case, FITC fluorescence band at 518 nm (Fig.4.1B) overlaps with the gold surface plasmon band at 527 nm (Fig.4.1A) and one expects effective energy transfer from the excited molecule to the gold surface. Hence, addition of spacer groups between the gold nanoparticles and FITC molecules to avoid energy transfer might provide a useful strategy to avoid rapid quenching of fluorescence emission from FITC. Lys and PLL were used as spacer groups between the gold nanoparticles and FITC molecules due the electrostatic and hydrogen bonding considerations mentioned earlier. The Au-lys-FITC and Au-PLL-FITC nanobioconjugates, when excited by 488 nm argon laser, showed considerably stable fluorescence and excellent confocal microscopy images (Fig.4.4H-I) could be routinely captured. The images recorded using CFLSM are in accordance with those observed by TEM, and further confirm the formation of large string like aggregates in case of Au-PLL-FITC and to a lesser extent in Au-lys-FITC.
nanobioconjugates. For the above reasons, Au-lys-FITC nanobioconjugates were used in further confocal microscopy experiments.

4.3.2 Endocytotic uptake of gold nanoparticles

In this part of the chapter, as-prepared borohydride reduced gold nanoparticles and Au-Lys-FITC nanoparticles were used for the internalization studies. Detailed studies of their uptake were performed employing Phase contrast microscopy, AFM, CFLSM, TEM and FACS. Furthermore, the mode of entry of nanoparticles was also studied using FACS. Macrophages are known to internalize solutes very rapidly, taking in the equivalent of their cell volume every 2 h [35]. It is therefore important to correlate any study on the cytotoxicity of gold nanoparticles with a detailed microscopic analysis about the pathway of particle uptake [33]. An understanding of the uptake of gold nanoparticles is also extremely important for drug and gene delivery applications.

We have used atomic force microscopy (AFM), confocal laser scanning microscopy (CFLSM) and transmission electron microscopy (TEM) to trace the time dependent kinetics of gold nanoparticle uptake inside the macrophage cells. The very first evidence of nanoparticles internalization into the cells comes from cell viability and phase contrast microscopy (Fig.4.5) wherein under mercury lamp nanoparticles accumulated inside the cells appear dark. Here it is noteworthy that the resolution of phase contrast microscope ranges in micrometer scale hence it is inferred that nanoparticles form larger aggregates after internalization. However it remains to be explored whether the aggregation takes place on cell surface or during the course of internalization. To clear our queries, the initial events of internalization were studied using AFM.

![Figure 4.5 Phase contrast image showing internalization of gold nanoparticles in macrophage cells. (Magnification 20 x)](image-url)
**AFM studies: Tracking early events of endocytosis**

AFM was used to study the initial stages of endocytosis. RAW264.7 macrophage cells were treated with 50 μM gold nanoparticle solution for different times as described in experimental section and were examined by tapping (Fig.4.6) and contact (Fig.4.7) mode AFM. Untreated cells were taken as a control.

Fig.4.6A and B show the tapping mode height (panel 1) and phase images (panel 2) of the control cells without treatment with gold nanoparticles. The untreated cells clearly show a part of a well-defined nucleus (towards the left side of the images) along with some regions of protruding cell membrane. The nucleus is clearly distinguishable from the cell membrane due to its greater height (lighter color signifies more height) than the cell membrane in the height image (Fig.4.6A.1), and higher contrast than that of the cell membrane in phase image (Fig.4.6A.2). The 3-D view (Fig.4.6A.3) of the height image further supports the information obtained in the Fig.4.6A.1 and A.2. The cell membrane architecture (Fig.4.6B) is clearly visible in the higher magnification image of the membrane region of the cells. Fig.4.6C and D show the tapping mode height and phase images of the cells treated with 50 μM gold nanoparticles for 5 min, again clearly exhibiting...
a part of well defined nucleus (towards the left side of the images Fig 4.6C.1 and C.2) along with some regions of protruding cell membrane. The nucleus is clearly distinguishable here from the cell membrane in the height image (Fig.4.6C.1), but in the phase image (Fig.4.6C.2) the cell membrane shows higher contrast than the nucleus, suggesting the presence of higher contrast gold nanoparticles on the cell surface but not on the nucleus. The 3-D view (Fig.4.6C.3) of the height image also clearly shows the presence of a well-defined nucleus. Fig.4.6D shows the region of cell membrane in greater detail. The higher contrast of the cell membrane region is clearly underlined in this image and is significantly higher than that of this region in the absence of gold nanoparticles (control in Fig.4.6B.2). Fig.4.7A and B show the contact mode height and friction images of the cells treated with 50 μM gold nanoparticles for 5 min. Fig.4.7A shows a well defined nucleus in the center of the images (clearly visible in the 3-D view in Fig.4.7A.3 as a dome-like structure) with a few nanopits (depth 25-70 nm).

The nucleus is surrounded by a large number of micropits (depth 150-300 nm) on the rest of the cell surface. Fig.4.7B shows higher magnification images of one of the micropits close to the nucleus (Fig.4.7A). The depth of the micropit shown in Fig.4.7B corresponds to 230 nm. The cellular domains present on the cell surface,
clearly visible in Fig. 4.7B.2, were found to be of dimensions ca. 300 nm and the gold nanoparticle aggregates present in the micropit shown in the same figure were found to be ca. 85 nm in size. It has been reported that pinocytosis occurs for particles smaller than 100 nm and phagocytosis for particles larger than 100 [52-53]. Since the gold nanoparticle used in this study range in size from 3-8 nm, involvement of the pinocytotic mechanism in gold nanoparticle uptake by cells appears to be operative. Raynal and co-workers have recently shown the uptake of superparamagnetic nanoparticles smaller than 100 nm via pinocytosis [36]. Moreover, the shape of the micropits in Fig.4.7A also suggests gold nanoparticle uptake via pinocytosis [1].

Endocytosis is a vital process for macrophage cells which leads to changes in surface and cytoskeletal architecture of the cells. After visualizing the endocytotic process of gold nanoparticle uptake, the logical succeeding step is to visualize the response of the cells in terms of regaining of the original cell surface architecture. To achieve this, a pulsed gold nanoparticle treatment was given to the macrophage cells for 15 min, followed by an overnight chase in nanoparticle-free media. Fig.4.7C and D show the contact mode height and friction images of the cells pulsed with 50 μM gold nanoparticles for 15 min followed by overnight chase. Both the height and friction mode images show a well-defined nucleus along with intact membrane. The interesting point is the absence of micro and nanopores that are clearly observed immediately after nanoparticle treatment (Fig.4.7A, B). Indeed, the images recorded after the overnight chase are on a gross level rather similar to the images obtained from the cells prior to gold exposure (Fig.4.6A, B). However, some effects of the exposure of the macrophage cells to the gold nanoparticles can be discerned in the higher magnification image of the cell membrane after pulse-chase gold nanoparticle treatment (Fig.4.7D). Cell surface structures appear to be generated by reconstitution of the micropits formed during the initial stages of endocytosis after gold nanoparticle treatment (Fig.4.7D.2).

**CFLSM studies: Gold nanoparticle reach inside the cells within the lysosomes in a perinuclear arrangement**

Confocal laser scanning microscopy (CFLSM) is one of the most important cellular analysis techniques and has been used with considerable success in the real-
time observation of cells. In this technique, molecular fluorescent probes are employed to label the cells to be observed. FITC is a versatile agent used for cell imaging by confocal microscopy [37]. After observing the initial steps of endocytosis by AFM (Fig. 4.6 and 4.7), confocal microscopy was used to study the kinetics of gold

Figure 4.8 Confocal microscopy images of RAW264.7 macrophage cells. (A-D) CFLSM images obtained after 30 min (A and B) and 3 h (C and D) of Au-lys-FITC treatment. A and C show the images taken using only fluorescent gold nanoparticles, while B and D show the images of internalized gold nanoparticles after lysosome staining. A1-A3 and C1-C3 show the fluorescent image, phase image, and fluorescent image overlapped with the phase image of the cells obtained after gold treatment for 30 min and 3 h, respectively. A4 shows a low magnification image of the cell shown in A1-A3. B1-B4 and D1-D4 show the lysotracker captured fluorescent image, FITC captured fluorescent image, phase image, and both lysotracker and FITC fluorescent image overlapped with the phase image of the cells obtained after gold nanoparticle treatment for 30 min and 3 h, respectively.
nanoparticle uptake at later stages of endocytosis. RAW264.7 macrophage cells treated with 50 μM Au·lys·FITC for 30 min and 3 h were observed by CFLSM. The overlapped image of the Fig.4.8A1 and A2 shown in Fig.4.8A3 suggests that these high contrast particles are gold nanoaggregates.

Untreated cells were taken as a control. Fig.4.8A and B show the confocal images of the cells obtained after treatment with gold nanoparticles for 30 min: Fig.4.8A1·A3 show the fluorescent image, phase image and fluorescent image overlapped with phase image of the cells respectively. The gold nanoaggregates can be clearly observed as green dots in the fluorescence image (Fig.4.8A1) and as high contrast particle aggregates in phase image (Fig.4.8A2). Fig.4.8A4 shows a lower magnification image of the structures shown in Fig.4.8A1·A3 clearly showing the complete macrophage cell with endocytosed gold nanoparticles. Lysosomes are the organelles in the general endocytosis pathway in which endocytosed particles enter and their fate is decided. We therefore used a lysotracker (a lysosome staining red fluorescent dye) to stain the lysosomes and then the subcellular localization of gold nanoparticles was traced using Au·lys·FITC. Fig.4.8B shows the images obtained by CFLSM after treatment of RAW264.7 macrophage cells with red lysotracker as well as green fluorescing gold nanoparticles. Fig.4.8B1 and B2 show the cells, observed after sequential scanning at 543 nm and 488 nm respectively. When excited using 543 nm laser, only the lysosomes are visible in the cells as red dots (Fig.4.8B1) while excitation by the 488 nm argon laser enables visualization of the gold nanoparticles.
in the cells as green dots (Fig. 4.8B2). Fig. 4.8B3 corresponds to the phase contrast image of the cells, showing the surface topology with a well-defined nucleus. Fig. 4.8B4 is the image obtained by overlaying Fig. 4.8B1-B3. It is clearly evident from Fig. 4.8B4 that the gold nanoaggregates are getting co-localized in the lysosomal bodies.

Fig. 4.8C and D correspond to confocal images of the cells after 3 h treatment with fluorescent gold nanoparticles, and fluorescent gold nanoparticles along with lysotracker, respectively. The images obtained in Fig. 4.8C and D are similar to those obtained in Fig. 4.8A and B respectively, except for the fact that after prolonged reaction of the macrophage cells with gold nanoparticles, the fluorescence from FITC-capped gold nanoaggregates and the lysotracker is observed closer to the nucleus. We note that no fluorescence from the nanoparticles could be observed inside the nucleus even after prolonged exposure to the cells. The confocal microscopy analysis (Fig. 4.8A-D) thus provides compelling evidence for cellular uptake of the gold nanoparticles, their internalization in lysosomes and time-dependent movement of these lysosomal bodies towards the nucleus before their final perinuclear arrangement – the gold nanoparticles do not enter the nucleus. However, negatively charged silica nanoparticles have recently been shown to enter inside the nucleus of the cells [38]. Therefore, endocytosis of various nanoparticles can not be generalized.

To investigate whether the gold nanoparticle uptake is a temperature dependent process, RAW264.7 cells were cultured with fluorescent gold nanoparticles under cold conditions (4 °C). We assume that this uptake would be inhibited at lower temperature, if it were performed via endocytotic pathways. As expected, we could not observe any fluorescent gold nanoparticles inside the cells (Fig. 4.9). In addition, after re-incubating the previously cooled cells at 37 °C with gold nanoparticles, their uptake by the cells studied by confocal microscopy was found to be quite facile. These results clearly show that the uptake of gold nanoparticles in macrophage cells occurs by an endocytotic pathway. Furthermore, the presence of fluorescent gold nanoparticles selectively in endosomes/lysosomes highlights the potential use of FITC-functionalized gold nanoparticles as biocompatible endosome/lysosome markers that function without activating or disrupting cell functions. This seems to be an advantage over semiconductor
quantum dots [39-40] that are commonly used as endosome/phagosome markers [41] that have been shown to disrupt cellular function as a consequence of their cytotoxic nature.

**TEM studies: Gold nanoparticles accumulate in lysosomes and do not enter into the nucleus**

The findings based on the AFM and CFLSM analysis was correlated with transmission electron microscopy (TEM) of the cells after cryotomy. TEM image of a cryotomed RAW264.7 macrophage cells treated with gold nanoparticles for 24 h is shown in Fig. 4.10. A part of the well-defined nucleus is visible towards the lower left end of the image. The presence of an intact nuclear membrane and other membrane structures strongly suggests that cell is viable and metabolically active. Gold nanoaggregates are clearly visible as higher contrast regions inside the lysosomes (dark patches identified by square-tipped arrows) while a few gold nanoparticle aggregates are observed outside other membrane structures. Among the various images, we could image a lysosome containing gold nanoaggregates lying immediately outside the nuclear pore (identified by a triangle-tipped arrow in Fig. 4.10) documenting its exact fate. Reticuloendothelial network in the form of endoplasmic reticulum can also be clearly seen emerging out from the nuclear pore and a few gold nanoparticle aggregates are visible in this reticuloendothelial network. Perinuclear arrangement of lysosomes is also clearly established by TEM and supports the AFM and confocal microscopy findings discussed earlier on the perinuclear arrangement of lysosomes containing gold nanoaggregates. It is interesting to note that even after 24 h of gold nanoparticle treatment, all the lysosomes containing gold nanoaggregates are observed only around the nucleus and not dispersed in the cytosol. It is well known
that antigenic proteins delivered to lysosomes via endocytosis are degraded to peptides, which then are recycled to endosomes containing recycling cell surface receptors, where they are associated with MHC class II molecules and their further processing takes place [42]. Gold nanoparticles might interfere with lysosomal enzymes involved in antigen processing due to their long-term accumulation in lysosomes or they may directly alter MHC molecules along the lysosome-endosome pathway and hence, these mechanisms could result in reduced production and presentation of self-peptides (arthritogens).

If these activities take place in conjunction with a redox system within phagocytic cells, as is suggested in this study by the reduction of RNS and ROS levels during prolonged and high gold nanoparticle concentration exposure (Chapter 3 Fig.2B-D), the anti-inflammatory action of gold can be effective over a prolonged period of time and thus of considerable value against autoimmune disorders. It is unlikely that the gold nanoparticles would be easily digested by lysosomal enzymes and therefore, the exocytosis of gold nanoparticles is not clear at this stage and could lead to bioaccumulation of gold nanoparticles inside the cellular compartments. Issues related to the clearance mechanism of gold nanoparticles from the cells are extremely important and need to be addressed in detail.

**FACS Studies: Mode of internalization of nanoparticles**

Nanoparticles have been considered as effective delivery vehicles, and have been studied extensively for the purpose of delivering drugs/genes into cells of interest [15]. In fact, targeted entry into cells is an important area of research in drug and gene delivery. Thus, site-specific delivery of drugs and therapeutics can significantly reduce the potential toxicity of a drug and increase its therapeutic effects. To maximize the efficiency of nanoparticle-mediated gene delivery, one should have detailed information regarding how the nanoparticles translocate into the cells. Presently, however, there are no rapid, simple and reproducible methods available for simultaneous measurement of cell surface attached nanoparticle and their internalization into the cells. In case of microorganism endocytosis, several methods including basic staining and light microscopy, electron microscopy, and radio-labeling or fluorophore conjugation to the microorganisms that allow differentiation between microorganisms simply attached and those internalized by
phagocytes have been described. Basic staining and microscopy techniques have major disadvantage that they are cumbersome and the interpretation is subjective [43-44] and also these techniques can not be applied in case of nanoparticles due their limited resolution. The electron microscopy is useful to show internalization, but serial sections are required to determine that nanoparticles are enclosed within a vacuole membrane. Radio-labeling techniques for internalization studies are limited due to safety measures [45-46].

Chaka and co-workers [47] have described a simple and rapid method for quantitation of internalized fluorescently labeled microorganisms; where they used trypan blue dye as a fluorescence quencher. In order to ascertain the mechanism of pathway of nanoparticle entry, we performed a study using macropinocytosis inhibitor cytochalasin B. Since it acts by inhibiting microfilament synthesis and membrane ruffling, cytochalasin B reversibly inhibits macropinocytosis [48, 51] and phagocytosis [49] in macrophages.

The assay was standardized using fluorescent latex beads (average bead size

![Figure 4.11](image_url)  
**Figure 4.11** Mode of endocytosis of gold nanoparticles. Curve 1 represents basal level of endocytosis. Curve 2- endocytosis of latex beads, curve 3- endocytosis of latex beads in presence of Cytochalasin B and curve 4- endocytosis of Au-Lys-FITC in presence of Cytochalasin B
0.8 µm) with or without preincubating RAW 264.7 macrophages in presence of cytochalasin B. Since pre-opsonized latex beads are known to be internalized via phagocytosis [50] we didn’t pre-opsonized the beads and used directly as received. Quenching of fluorescence of beads and Au·Lys·FITC nanoparticles was confirmed by incubating with 0.02 % trypan blue solution for 10 min followed by observation under fluorescent microscope. Figure 4.11 represents the mode of endocytosis of gold nanoparticles. Curve 1 corresponds to the basal level of endocytosis in normal untreated control cells. The cells representing curve 1 were exposed to fluorescent beads for 2h. The appearance of an additional peak in FL·1 region in curve 2 represents the increase in fluorescence as indicative of phagocytosed fluorescent beads. Since the cells were neither starved nor stimulated the second peak represents the basal level of endocytosis of beads. In any cell population at a given time not all the cells are in the same phase of differentiation so the differences in their endocytosis capacity. The reduction in the height of curve 2 is due to the shift of only a fraction of cells with endocytic activity towards increased fluorescence. This shift in the peak could be blocked (curve 3) by incubating the cells with 2 µM cytochalasin B for 90 min prior to exposure of beads. Cytochalasin B is a known inhibitor of phagocytosis and the same inhibitory dose has been used by others [51–52] in case of murine peritoneal macrophages. The same inhibitory concentration of 2 µM cytochalasin B was used to study Au·Lys·FITC. The cells were pretreated with 2 µM cytochalasin B followed by 2h incubation with fluorescent gold nanoaggregates (curve 4). Interestingly, we observed that gold nanoparticles do get internalized into the cells even in presence of an inhibitor at a concentration as high as 5 µM. Thus the results obtained clearly indicate that the nanoaggregates do not get internalized via macropinocytosis as well as phagocytosis. These results do not support our previous statement where on the basis of the size of nanopits measured by AFM we hypothesized the macropinocytotic mode of particle internalization. However, Rejman and co-workers [53] have shown that ≥ 200nm sized nanoparticles can be internalized via caveolin mediated pathway. Further experimentations in the presence of mode specific inhibitors will be useful to shed a light on the exact mode of internalization. Nanoparticles display very large surface to volume ratio. In a recent report, Cedervall and co-worker [54], employing tailored co polymers in tandem with techniques such as isothermal calorimetry and size exclusion
chromatography, have elegantly demonstrated that nanoparticle size, hydrophobicity and protein identity all contribute to nanoparticle protein interaction in vitro and possibly in vivo. It is possible that during the course of cellular internalization, nanoparticles encounter different proteins and their interaction with biomolecules leads to surface modification and thus under different conditions employ more than one mode of internalization.

4.5 Conclusion

This investigation demonstrates surface functionalization of gold nanoparticles using amino acids and low molecular weight polypeptides followed by fluorescent conjugation of functionalized nanoparticles with FITC. These functionalized nanoparticles are nontoxic to cells. Au·FITC nanoparticles are readily quenched due to overlapping emission band of FITC and absorption band of AuNP. Introduction of lysine and PLL as spacers inhibits quenching of fluorescence. Both, Au·Lys·FITC and Au·PLL·FITC exhibit excellent fluorescence. In addition, we have shown the antioxidant effect of gold nanoparticles and the path of their eventual internalization in perinuclearly arranged lysosomes. These findings have implications in the design of effective targeted drug/gene delivery systems. The biocompatible fluorescent systems, employed as beacons for the nanoparticles in the present study, are promising for combining cancer imaging and tumor-targeted drug delivery in cancer therapy. Further, endocytosis of gold nanoparticles in presence of mode specific inhibitor Cytochalasin B suggests that nanoparticles possibly get internalized in cells via more than one pathway.
Chapter 4

4.6 References


